DETOXIFIED MUTANTS OF BACTERIAL ADP-RIBOSYLATING TOXINS AS PARENTERAL ADJUVANTS

Cross-Reference to Related Application

This application is related to provisional patent application serial no. 60/041,227, filed March 21, 1997, from which priority is claimed under 35 USC §119(e)(1) and which is incorporated herein by reference in its entirety.

Field of the Invention

The present invention relates to adjuvants useful for the administration of antigens to organisms. In particular, the adjuvants of the invention allow the parenteral delivery of vaccines to raise an immune response.

Background of the Invention

Advances in recombinant DNA technology have made possible the generation of a variety of vaccines, such as subunit vaccines and DNA-based vaccines. These are in addition to the more traditional killed or attenuated vaccines. Adjuvants that enhance the immune system's response to antigenic material are known; however, currently, few adjuvants are approved for human usage, although additional adjuvants are in pre-clinical and clinical studies.

The ADP-ribosylating bacterial toxins, a group of potent toxins to humans, include diphtheria toxin, pertussis toxin (PT), cholera toxin (CT), the *E. coli* heat-labile toxins (LT1 and LT2), *Pseudomonas* endotoxin

A, *C. botulinum* C2 and C3 toxins, as well as toxins from *C. perfringens*, *C. spiriforma* and *C. difficile*. These toxins are composed of a monomeric, enzymatically active A subunit which is responsible for ADP-ribosylation of GTP-binding proteins, and a non-toxic B subunit which binds receptors on the surface of the target cell and delivers the A subunit across the cell membrane.

In the case of CT and LT, the A subunit is known to increase intracellular cAMP levels in target cells, while the B subunit is pentameric and binds to GM1 ganglioside receptors. (LT-B also binds additional receptors.)

Previously, observations were made demonstrating that CT is able to induce mucosal and systemic immunity against itself when administered intraduodenally (i.e. to a mucosal surface). The membrane-binding function of CT was shown to be essential for mucosal immunogenicity, but cholera toxoid, also known as the B subunit of CT (CT-B) was inactive in isolation (Pierce and Gowans, *J. Exp. Med.* 1975; 142: 1550; Pierce, *J. Exp Med.* 1978; 148: 195-206).

Subsequently, it was demonstrated that native CT induced immunity to co-administered antigens (Elson, *Curr. Top. Microbiol. Immunol.*, 1989; 146:29; Elson and Ealding, *J. Immunol.* 1984; 133:2892-2897; Elson and Ealding, *Ibid.* 1984; 132:2736-2741; Elson et al., *J. Immunol. Meth.* 1984; 67:101-118; Lycke and Homgren, *Immunology* 1986; 59:301-339) and that immune responses may be elicted to diptheria or tetanus toxoids when these antigens are applied to skin in combination with CT.

Two approaches have been taken in order to address the problem of CT toxicity. The first approach has involved the use of CT-B as a mucosal adjuvant. CT-B is entirely non-toxic. An adjuvant effect for co-administered CT-B has been alleged in a number of

publications (Tamura et al., *J. Immunol.* 1992; 149:981-988; Hirabayashi et al., *Immunology* 1992; 75: 493-498; Gizurarson et al., *Vaccine* 1991; 9:825-832; Kikuta et al., *Vaccine* 1970; 8:595-599; Hirabayashi et al. *Ibid.* 1990; 8:243-248; Tamura et al., *Ibid.* 1989; 7:314-32-; Tamura et al., *Ibid.* 1989; 7:257-262; Tamura et al., *Ibid.* 1988; 6:409-413; Hirabayashi et al., *Immunology* 1991; 72:329-335 Tamura et al., *Vaccine* 1989; 7:503-505).

However, a number of aspects of the observations reported above were not entirely convincing. For example, it was noted that the adjuvant effect ascribed to CT-B was not H-2 (MHC) restricted. It is known, however, that the immune response to CTB is H-2 (MHC) restricted (Elson and Ealding, *Eur. J. Immuno.* 1987; 17:425-428). Moreover, the alleged adjuvant effect was observed even in individuals already immune to CT-B.

Other groups were unable to observe any mucosal adjuvant effect attributable to CT-B (Lycke and Holmgren, *Immunology* 1986; 59:301-308; Lycke et al., *Eur. J. Immunol.* 1992: 22:2277-2281). Experiments with recombinant CT-B (Holmgren et al., *Vaccine* 1993; 11:1179-1183) confirmed that the alleged effect is largely, if not exclusively, attributable to low levels of contamination of CT-B preparations with CT.

A second approach to eliminating the toxicity of CT has been to mutate the active holotoxin in order to reduce or eliminate its toxicity. The toxicity of CT resides in the A subunit and a number of mutants to CT and its homologue, LT, comprising point mutations in the A subunit, are known in the art. See, for example, International Patent Application W092/19265. It is accepted in the art that CT and LT are generally interchangeable, showing considerable homology. ADP-ribosylating bacterial toxin mutants have been shown to

act as mucosal adjuvants, see International Patent Application W095/17211.

Summary of the Invention

Accordingly, there remains a need for an active adjuvant which may be used to increase the immunogenicity of an antigen when administered parenterally, such as intramuscularly, subcutaneously, intravenously, transcutaneously or intradermally. The present invention provides for such parenteral adjuvants in the form of non-toxic ADP ribosylating bacterial toxins. It is demonstrated herein that such mutants, lacking toxicity, are active as parenteral adjuvants and produce high antibody titers and/or induction of cytotoxic T-lymphocytes (CTLs).

In one embodiment, then, the subject invention is directed to a parenteral adjuvant composition comprising a detoxified mutant of a bacterial ADP-ribosylating toxin as the parenteral adjuvant and at least one selected antigen.

In another embodiment, the invention is directed to a parenteral adjuvant composition comprising a detoxified mutant of a bacterial ADP-ribosylating toxin as the parenteral adjuvant and a pharmaceutically acceptable topical vehicle.

In yet another embodiment, the invention is directed to a parenteral adjuvant composition comprising a detoxified mutant of a bacterial ADP-ribosylating toxin as the parenteral adjuvant, a pharmaceutically acceptable topical vehicle and at least one selected antigen.

In another embodiment, the invention is directed to a method for making a parenteral adjuvant composition comprising combining a detoxified mutant of a bacterial ADP-ribosylating toxin as the parenteral adjuvant with at least one selected antigen.

In still a further embodiment, the invention is directed to a method of making a parenteral adjuvant composition comprising combining a detoxified mutant of a bacterial ADP-ribosylating toxin as the parenteral adjuvant with a pharmaceutically acceptable topical vehicle.

In another embodiment, the invention is directed to a method for immunizing a vertebrate subject comprising parenterally administering to the vertebrate subject an immunologically effective amount of

a) an adjuvant comprising a detoxified mutant of a bacterial ADP-ribosylating toxin in combination with a pharmaceutically acceptable vehicle; and

b) at least one selected antigen.

In particularly preferred embodiments, the non-toxic adjuvant is a detoxified mutant selected from the group consisting of cholera toxin (CT), pertussis toxin (PT), and an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, CT-S109, and PT-K9/G129.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figures 1A-1B show the DNA and corresponding amino acid sequences of a wild-type subunit A from an *E. coli* heat labile toxin (LT) (SEQ ID NOS:1 and 2) and a cholera toxin (CT) (SEQ ID NOS:3 and 4).

Figure 2 shows the serum anti-LT antibody response following transcutaneous administration of representative adjuvant compositions of the present invention. Circles indicate titers from individual mice. If less than five circles are visible per group, two or more values were identical and circles were superimposed.

Full triangles indicate mean titers per group ± standard deviation.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989); DNA CLONING, VOLUMES I AND II (D.N Glover ed. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), METHODS IN ENZYMOLOGY Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), Mayer and Walker, eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir and C. C. Blackwell eds 1986).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include

plural references unless the content clearly dictates otherwise.

The following amino acid abbreviations are used throughout the text:

Alanine (Ala)	A	Arginine (Arg)	R
Asparagine (Asn)	N	Aspartic acid (Asp)	D
Cysteine (Cys)	C	Glutamine (Gln)	Q
Glutamic acid (Glu)	E	Glycine (Gly)	G
Histidine (His)	H	Isoleucine (Ile)	I
Leucine (Leu)	L	Lysine (Lys)	K
Methionine Met)	M	Phenylalanine (Phe)	F
Proline (Pro)	P	Serine (Ser)	S
Threonine (Thr)	T	Tryptophan (Trp)	W
Tyrosine (Tyr)	Y	Valine (Val)	V

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "parenteral" is meant introduction into the body outside of the digestive tract, such as by subcutaneous, intramuscular, transcutaneous, intradermal, or intravenous administration. This is to be contrasted with adjuvants that are delivered to a mucosal surface, such as oral, intranasal, vaginal, or rectal.

As used herein, "detoxified" refers to both completely nontoxic and low residual toxic mutants of the toxin in question. Preferably, the detoxified protein retains a toxicity of less than 0.01% of the naturally occurring toxin counterpart, more preferably less than 0.001% and even more preferable, less than 0.0001% of the toxicity of the naturally occurring toxin counterpart. The toxicity may be measured in mouse CHO cells or preferably by evaluation of the morphological changes

induced in Y1 cells. In particular, Y1 cells are adrenal tumor epithelial cells which become markedly more rounded when treated with a solution containing CT or LT (Ysamure et al., *Cancer Res.* (1966) 26:529-535). The toxicity of CT and LT is correlated with this morphological transition. Thus, the mutant toxins may be incubated with Y1 cells and the morphological changes of the cells assessed.

The term "toxoid" as used herein means a genetically detoxified toxin.

By "antigen" is meant a molecule which contains one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a cellular antigen-specific immune response when the antigen is produced, or a humoral antibody response. Such epitopes may include from about 3 to about 20 or more amino acids. Normally, a B cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids, and a helper T cell epitope at least about 12-20 amino acids. The term denotes both subunit antigens, i.e., antigens which are separate and discrete from a whole organism with which the antigen is associated in nature, as well as killed, attenuated or inactivated bacteria, viruses, parasites or other microbes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein.

For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi. The term also intends any of the various tumor antigens. Furthermore, for purposes of the present invention, an "antigen" refers to a

protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to molecules present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. T cells can be divided into two major groups called $CD8^+$ T or $CD4^+$ T cells based on expression of either the CD8 or CD4 protein on their surface. $CD8^+$ T cells are often referred to as cytotoxic T cells (CTL) and $CD4^+$ T cells are often called helper T cells (Th). The Th cells can be further divided into Th1 and Th2 cells. In contrast to the B cells, T cells are not capable of recognizing native antigen but require specific processing of such antigens. Fragments of the antigen are presented by antigen presenting cells (APCs) to T cells. These fragments are associated with a specific protein on the surface of the APC. $CD8^+$ T cells recognize the fragment presented by MHC I protein whereas $CD4^+$ T cells recognize antigenic fragments presented by MHC II proteins.

One important aspect of cellular immunity involves an antigen-specific response by CTLs. CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the

surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T cells. Helper T cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T cells and/or other white blood cells, including those derived from CD4+ and CD8+ T cells.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376; and the examples below.

Thus, an immunological response as used herein may be one which stimulates the production of CTLs, and/or the production or activation of helper T cells. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an

immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or $\gamma\delta$ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art. For a general overview of the immune system and immunological mechanisms see for example: Janeway, C.A. and Travers, P., IMMUNOBIOLOGY, 2nd ed. 1996, Current Biology Ltd./Garland Publishing, New York, NY.

A composition which contains a selected antigen along with a detoxified mutant of a bacterial ADP-ribosylating toxin of the present invention, or a vaccine composition which is coadministered with the subject adjuvant, displays "enhanced immunogenicity" when it possesses a greater capacity to elicit an immune response than the immune response elicited by an equivalent amount of the antigen administered without the adjuvant. Thus, a vaccine composition may display "enhanced immunogenicity" because the antigen is more strongly immunogenic or because a lower dose or fewer doses of antigen are necessary to achieve an immune response in the subject to which the antigen is administered. Such enhanced immunogenicity can be determined by administering the adjuvant composition and antigen controls to animals and comparing antibody titers and/or cellular-mediated immunity against the two using standard assays such as radioimmunoassay, ELISAs, CTL assays, and the like, well known in the art.

For purposes of the present invention, an "effective amount" of an adjuvant will be that amount which enhances an immunological response to a coadministered antigen.

As used herein, "treatment" refers to any of (i) prevention of infection or reinfection, as in a traditional vaccine, (ii) reduction or elimination of symptoms, and (iii) reduction or complete elimination of the pathogen in question. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

By "vertebrate subject" is meant any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like; and fish. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

II. Modes of Carrying Out the Invention

The present invention is based on the surprising and unexpected discovery that detoxified mutants of bacterial ADP-ribosylating toxins, such as CT, LT and PT, are able to serve as parenteral adjuvants to enhance humoral and/or cell-mediated immune responses in a vertebrate subject when the adjuvants are administered with a selected antigen. Since the present adjuvants

function when administered parenterally, they permit a convenient method of conferring immunity to substances that are not amenable to other modes of administration. Accordingly, the present system is useful with a wide variety of antigens and provides a powerful tool to prevent and/or treat a large number of infections.

Regarding the present invention, any detoxified mutant of a bacterial ADP-ribosylating toxin can be used as a parenteral adjuvant. Such mutants optionally comprise one or more amino acid additions, deletions or substitutions that result in a molecule having reduced toxicity while retaining adjuvanticity. If an amino acid is substituted for the wild-type amino acid, such substitutions may be with a naturally occurring amino acid or may be with a modified or synthetic amino acid. Substitutions which alter the amphotericity and hydrophilicity while retaining the steric effect of the substituting amino acid as far as possible are generally preferred.

The mutants of the invention are preferably in the form of a holotoxin, comprising the mutated A subunit and the B subunit, which may be oligomeric, as in the wild-type holotoxin. The B subunit is preferably not mutated. However, it is envisaged that a mutated A subunit may be used in isolation from the B subunit, either in an essentially pure form or complexed with other agents, which may replace the B subunit and/or its functional contribution.

As explained above, in addition to the completely nontoxic ADP-ribosylating bacterial toxins, toxins can be used wherein a residual toxicity greater than 100 to 10,000 fold lower, or more, than its naturally occurring counterparts is found.

Particularly suitable are detoxified mutants of diphtheria toxin, CT, LT, or PT; such mutations are known

in the art. For example, particular mutant LTs in accordance with the invention may possess the following mutations of the A subunit: a Val to Asp, Glu or Tyr substitution at position 53; a Ser to Lys substitution at position 63 (termed LT-K63 herein); an Ala to Arg substitution at position 72 (termed LT-R72 herein); a Val to Lys or Tyr substitution at position 97; a Tyr to Lys, Asp or Ser substitution at position 104; a Pro to Ser substitution at position 106; an Arg to Gly substitution at position 192.

Since the amino acid sequences of CT-A and LT-A are substantially conserved (see Figures 1A-1B, (SEQ ID NOS:1-4)), the changes described above with respect to LT can also be made to the corresponding positions in CT. A particularly preferred CT mutant comprises a substitution of Ser at position 109 (termed CT-S109 herein).

A preferred detoxified mutant of *Bordetella pertussis* is a double mutant where Lys replaces Arg at amino acid position 9 and Gly replaces Glu at amino acid position 129 (termed PT-K9/G129 herein). Many other suitable pertussis toxin (PT) mutants are known in the art.

Methods for the design and production of mutants of CT and/or LT are known in the art. Suitable methods are described in International Patent application W093/13202, as well as W092/19265. In particular, such mutant toxins may be synthesized chemically, using conventional peptide synthesis techniques. See, e.g., See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of*

Peptide Synthesis, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology, supra*, Vol. 1, for classical solution synthesis.

Alternatively, and preferably, mutations can be made to the wild-type sequence using conventional recombinant techniques such as by preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding the wild-type protein using restriction endonuclease digestion. (See, e.g., Kunkel, T.A. *Proc. Natl. Acad. Sci. USA* (1985) 82:448; Geisselsoder et al. BioTechniques (1987) 5:786.) Alternatively, the mutations can be effected using a mismatched primer which hybridizes to the wild-type nucleotide sequence (generally cDNA corresponding to the RNA sequence), at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by keeping the mutant base centrally located. Zoller and Smith, *Methods Enzymol.* (1983) 100:468. Primer extension is effected using DNA polymerase, the product cloned and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Selection can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations. See, e.g., Dalbie-McFarland *et al. Proc. Natl. Acad. Sci USA* (1982) 79:6409. PCR mutagenesis will also find use for effecting the desired mutations.

The adjuvant of the invention is preferably administered in admixture with at least one antigen against which it is desired to raise an immune response. If the antigen and the adjuvant are not in admixture, it is preferred that they be administered within a

relatively short time of each other, at the same site of administration, although there may be a delay of up to 5 days and a two-injection site regime. Thus, the adjuvant may be administered prior or subsequent to, or concurrent with the selected antigen. It has been observed that the adjuvant effect provided by wild-type CT is short-lived (see Lycke and Homgren, *Immunology* 1986: 59: 301-308).

In an alternative embodiment, the adjuvant of the present invention may be administered, optionally in isolation from other antigens, as a boost following systemic or mucosal administration of a vaccine.

Diseases against which the subject may be immunized include all diseases capable of being treated or prevented by immunization, including viral diseases, allergic manifestations, diseases caused by bacterial or other pathogens, such as parasitic organisms, AIDS, autoimmune diseases such as Systemic Lupus Erythematosus, Alzheimer's disease and cancers. Vaccine formulations suitable for delivery may be prepared as set out hereinbelow, while further formulations will be apparent to those of still in the art.

Thus, the antigen may be any antigen to which it is desired to raise an immune response in the subject. Suitable antigens comprise bacterial, viral, fungal and protozoan antigens derived from pathogenic organisms, as well as allergens, and antigens derived from tumors and self-antigens. Typically, the antigen will be a protein, polypeptide or peptide antigen, but alternative antigenic structures, such as nucleic acid antigens, carbohydrate antigens and whole or attenuated or inactivated organisms such as bacteria, viruses or protozoa are included.

Specific examples of antigens useful in the present invention include a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1

and HSV-2 glycoproteins gB, gD and gH; antigens derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7. (See, e.g. Chee et al., *Cytomegaloviruses* (J.K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et al., *J. Gen. Virol.* (1988) 69:1531-1574, for a discussion of the various HSV-1 encoded proteins; U.S. Patent No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., *Nature* (1984) 310:207-211, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, *J. Gen. Virol.* (1986) 67:1759-1816, for a review of VZV.)

The adjuvant compositions of the present invention can also be used to deliver antigens from the hepatitis family of viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV). By way of example, the viral sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including E1 (also known as E) and E2 (also known as E2/NSI). (See, Houghton et al., *Hepatology* (1991) 14:381-388, for a discussion of HCV proteins, including E1 and E2.) The sequences of these proteins, as well as antigenic fragments thereof, will find use in the present methods. Similarly, the sequence for the δ-antigen from HDV is known (see, e.g., U.S. Patent No. 5,378,814) and this antigen can also be conveniently used in the present methods.

Antigens derived from other viruses will also find use in the claimed methods, such as without limitation, proteins from members of the families Picornaviridae (e.g., polioviruses, etc.); Caliciviridae; Togaviridae (e.g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabodoviridae (e.g., rabies virus, etc.); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae (e.g., influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviradae (e.g., HTLV-I; HTLV-II; HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV_{IIIb}, HIV_{SF2}, HIV_{LAV}, HIV_{LAI}, HIV_{MN}); HIV-1_{CM235}, HIV-1_{US4}; HIV-2; simian immundeficiency virus (SIV) among others. See, e.g. Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991), for a description of these and other viruses.

For example, the present adjuvants can be used in conjunction with the gp120 envelope protein from HIV_{SF2}, HIV-1_{CM235}, HIV-1_{US4}, HIV-1_{IIIB} and HIV-1_{LAI}. The gp120 sequences for these and a multitude of additional HIV-1 and HIV-2 isolates, including members of the various genetic subtypes of HIV, are known and reported (see, e.g., Myers et al., Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico (1992); Myers et al., *Human Retroviruses and Aids, 1990*, Los Alamos, New Mexico: Los Alamos National Laboratory; and Modrow et al., *J. Virol.* (1987) 61:570-578, for a comparison of the envelope sequences of a variety of HIV isolates) and sequences derived from any of these isolates will find use in the present methods. Furthermore, the invention is equally applicable to other immunogenic proteins derived from any of the various HIV isolates, including

any of the various envelope proteins such as gp160 and gp41, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol region.

Additionally, the envelope glycoproteins HA and NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology* (1990) 179:759-767; Webster et al., "Antigenic variation among type A influenza viruses," p. 127-168. In: P. Palese and D.W. Kingsbury (ed.), *Genetics of influenza viruses*. Springer-Verlag, New York). Thus, proteins derived from any of these isolates can also be used in the techniques described herein.

The compositions and methods described herein will also find use with numerous bacterial antigens, such as those derived from organisms that cause diphtheria, cholera, tuberculosis, tetanus, pertussis, meningitis, and other pathogenic states, including, without limitation, *Bordetella pertussis*, *Neisseria meningitides* (A, B, C, Y), *Hemophilus influenza* type B (HIB), and *Helicobacter pylori*. Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

Furthermore, the methods and compositions described herein provide a means for treating a variety of malignant cancers. For example, the system of the present invention can be used to mount both humoral and cell-mediated immune responses to particular proteins specific to the cancer in question, such as an activated oncogene, a fetal antigen, or an activation marker. Such tumor antigens include any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc. (Boon, T. *Scientific American* (March 1993):82-89); any of the various tyrosinases; MART 1 (melanoma antigen recognized by T cells), mutant ras; mutant p53; p97

melanoma antigen; CEA (carcinoembryonic antigen), among others.

It is readily apparent that the subject invention can be used prophylactically (to prevent disease) or therapeutically (to treat disease after infection) for a wide variety of diseases. Not only are the compositions herein useful for preventing or treating disease, the subject compositions may also be used to prepare antibodies, both polyclonal and monoclonal, useful for, e.g., diagnostic purposes, as well as for immunopurification of particular antigens against which they are directed.

If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with the adjuvant compositions of the present invention, along with the desired antigen. In order to enhance immunogenicity, the antigen can be linked to a carrier prior to immunization. Immunization for the production of antibodies is generally performed by injecting the composition parenterally (generally subcutaneously or intramuscularly). The animal is usually boosted 2-6 weeks later with one or more injections of the antigen, with the adjuvant compositions described herein or with alternate adjuvants. Antibodies may also be generated by *in vitro* immunization, using methods known in the art. Polyclonal antisera is then obtained from the immunized animal and treated according to known procedures. See, e.g., Jurgens et al. (1985) *J. Chrom.* 348:363-370.

Monoclonal antibodies are generally prepared using the method of Kohler and Milstein, *Nature* (1975) 256:495-96, or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is

removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of non-specifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B cells, expressing membrane-bound immunoglobulin specific for the antigen, will bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice). See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980); Hammerling *et al.*, *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the hormone of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc.

Compositions according to the invention may comprise one or more antigens. Furthermore, one or more "pharmaceutically acceptable excipients or vehicles" are present, such as water, saline, glycerol, polyethyleneglycol, hyaluronic acid, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

A carrier is optionally present that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as further immunostimulating agents ("adjuvants").

Typically, the compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. Furthermore, compositions suitable for topical use may also be formulated. For example, the adjuvant compositions may be provided in the form of pharmaceutically acceptable topical vehicles such as ointments, creams, gels and emulsions. Ointments, creams and emulsions containing the adjuvants can be prepared using known techniques. A variety of suitable pharmaceutical ointment bases are generally known, including oleaginous bases, anhydrous absorption bases, and oil-in-water (o/w) bases. Oleaginous bases include petrolatum or petrolatum modified by waxes (e.g., liquid petrolatum gelled by the addition of a polyethylene) and those prepared from vegetable fixed oils or animal fats (e.g., lard, benzoinated lard, olive oil, cottonseed oil, or the like). Anhydrous bases include hydrophilic petrolatum, anhydrous lanolin and lanolin derivatives. Oil-in-water bases (e.g., emulsion bases or creams) generally include three parts, the oil phase, the

emulsifier and the aqueous phase. The adjuvant, and optionally the antigen, can be included in any one of the phases, or added to the formed emulsion. The oil phase is typically comprised of petrolatum with one or more higher molecular weight alcohols such as cetyl or steryl alcohol. The aqueous phase generally contains preservatives, the water-soluble components of the emulsion system, humectants (such as glycerin, propylene glycol or a polyethylene glycol), as well as optional stabilizers, antioxidants, buffers and the like.

The above pharmaceutical ointments are formed by dispersing finely divided or dissolved adjuvant and, optionally one or more selected antigens, uniformly throughout the vehicle or base. Creams, lotions and emulsions can be formed by way of a two-phase heat system, wherein oil-phase ingredients are combined under heat to provide a liquified, uniform system. The aqueous-phase ingredients are separately combined using heat. The oil and aqueous phases are then added together with constant agitation and allowed to cool. At this point, concentrated agents may be added as a slurry. Volatile or aromatic materials can be added after the emulsion has sufficiently cooled. Preparation of such pharmaceutical compositions is within the general skill of the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

The adjuvants can also be incorporated into gel formulations using a two-phase gel system. Such systems generally comprise a suspension or network of small, discrete particles interpenetrated by a liquid to provide a dispersed phase and a liquid phase. Single-phase gel systems are formed by distributing organic macromolecules uniformly throughout a liquid such that there are no apparent boundaries between the dispersed and liquid

phases. Suitable gelling agents for use herein include synthetic macromolecules (e.g., Carbomers®, polyvinyl alcohols and polyoxyethylene-polyoxypropylene copolymers), gums such as tragacanth, as well as sodium alginate, gelatin, methylcellulose, sodium carboxymethylcellulose, methylhydroxyethyl cellulose and hydroxyethyl cellulose. In order to prepare a uniform gel, dispersing agents such as alcohol or glycerin can be added, or the gelling agent can be dispersed by trituration, mechanical mixing or stirring, or combinations thereof.

Lotion preparations are generally liquid or semiliquid preparations containing the adjuvant and, optionally, one or more selected antigens, in a suitable vehicle. Lotions are formed by suspending finely divided solids in an aqueous medium. Optional dispersing agents can be employed to aid in the preparation of the liquid formulation, as well as one or more surface-active agents.

In the cream and ointment formulations described hereinabove, optional ingredients can include materials such as antioxidants, viscosity modifiers (e.g., paraffin wax or lanolin wax), and topical absorption rate modifiers. Actual methods of preparing any of the above formulations are known, or will be apparent, to those skilled in the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the adjuvant and an antigen, as well as any other the above-mentioned components, as needed. By "immunologically effective amount", is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is such that an immune response can

be generated in the subject to which it is administered. The exact amount necessary will vary depending on the subject being treated; the age and general condition of the subject to be treated; the capacity of the subject's immune system to synthesize antibodies and/or mount a cell-mediated immune response; the degree of protection desired; the severity of the condition being treated; the particular antigen selected and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. Thus, an "immunologically effective amount" will fall in a relatively broad range that can be determined through routine trials. In general, an "immunologically effective" amount of antigen will be an amount on the order of about 0.1 μg to about 1000 μg, more preferably about 1 μg to about 100 μg.

Similarly, the adjuvant will be present in an amount such that the antigen displays "enhanced immunogenicity," as defined above, as compared to administration of the antigen alone, without the adjuvant. Amounts which are effective for eliciting an enhanced immune response can be readily determined by one of skill in the art.

Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

Additional adjuvants can be used to enhance effectiveness; such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO

90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, KA), (b) SAF, containing 10%- Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon ™ (Cambridge Rioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g., gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred. Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-Disoglutamine (thr-MDP), N-acetyl-normuramyl-^{L}-alanyl-^{D}-isoglutamine (nor-MDP) , N-acetylmuramyl-^{L}-alanyl-^{D}-isoglutaminyl-^{L}-alanine-2- (1'-2'-dipalmitoyl-*sn*-glycero-3 - huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

The invention further provides a method for the manufacture of an adjuvanted vaccine comprising the steps of:

a) performing site-directed mutagenesis on the A subunit of a bacterial ADP-ribosylating toxin in order to detoxify the toxin; and

b) combining the detoxified toxin with an antigen, such that it functions as a parenteral adjuvant.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1

Production of LT-K63

LT-K63, for use in the following experiments, was made as follows. Site-directed mutagenesis was used to replace the Ser reside at position 63 of the A subunit of LT with Lys in order to construct a non-toxic LT mutant that would still assemble as a holotoxin with cell binding activity. The mutant protein, named LT-K63, was purified and tested for ADP-ribosyltransferase and toxic activity in several assays. LT-K63 was still able to bind GM1 ganglioside receptor but showed a complete loss of enzymatic activity, in agreement with published data (Lobet et al., *Infect. Immun.* 1991; 59:2870-2879). Further, LT-K63 was inactive in the ~~mouse~~ rabbit ileal loop assay and *in vitro* on Y1 cells.

Example 2

Parenteral adjuvant activity of LT-K63 with HSV gD2 in mice

The LT-K63 mutant, produced as described in Example 1, was tested as a parenteral adjuvant with herpes simplex virus type 2 (HSV-2) gD antigen as follows.

a. Mice were immunized twice by intramuscular injection one month apart with a 10 μg dose of the LT-K63 mutant and a 10 μg dose of HSV-2 gD2 antigen. Sera were collected on day 0 and two weeks after the second immunization (day 42). The antibody responses against HSV-2 gD2 were measured by ELISA. The geometric mean titers plus or minus standard error are listed in Table 1. This experiment illustrates the ability of the LT-K63 mutant in combination with HSV-2 gD2 to produce an immune response in mice.

Table 1. Serum anti-gD2 titers of mice immunized with LT-K63 and gD2		
Animal #	Day 0	Day 42
BL898	<10	4510
BL899	<10	45920
BL900	<10	7535
BL901	<10	56585
BL902	<10	74085
BL903	<10	8845
BL904	<10	4340
BL905	<10	19430
BL906	<10	6380
BL907	<10	9125
GMT +/- SEM		13975 +/- 4726

b. Mice were immunized twice by intramuscular injection one month apart with 10 μg HSV-2 gD2. Sera were collected on day 0 and two weeks after the second immunization. The antibody responses against HSV-2 gD2 were measured by ELISA and are shown as geometric mean titer plus or minus standard error in Table 2. The 90-fold lower antibody response produced by HSV-2 gD2 (150) compared to HSV-2 gD2 combined with LT-K63 (13980) illustrates the parenteral adjuvant activity of LT-K63 with HSV-2 gD2.

Table 2. Serum anti-HSV gD2 antibody titers of mice immunized with gD2		
Animal #	Day 14 Sera	Day 42 Sera
BC047	<10	348
BC048	<10	11
BC049	<10	17
BC050	<10	222
BC051	<10	73
BC052	<10	44
BC053	<10	2053
BC054	<10	2882
GMT +/- SEM		151 +/- 111

Example 3

Parenteral adjuvant activity of LT-K63 with influenza HA in mice

Mice were immunized twice by intramuscular injection one month apart with 1 μg LT-K63 (produced as described in Example 1) and 1 μg A/Texas HA (hemagglutinin) antigen or 1 μg A/Texas HA alone. Sera were collected two weeks after the second immunization. The anti-HA ELISA titers are shown as geometric mean titer plus or minus standard error in Table 3. The 11-fold higher antibody response observed in the group receiving HA antigen combined with the LT-K63 mutant (70380) compared with the group receiving HA antigen alone (6390) illustrates the effectiveness of LT-K63 as a parenteral adjuvant with influenza HA antigen.

Table 3. Serum anti-HA titers of mice immunized with HA or HA with LT-K63				
HA Animal #	Day 42		HA + LT-K63 Animal #	Day 42
CN 622	3698			
CN 623	7778			
CN 624	5506			
CN 625	5142			
CN 626	7109			
CN 627	7422			
CN 628	51463			
CN 629	19299			
CN 630	2906			
CN 631	427			
CN 632	2601		CN 642	73486
CN 633	4817		CN 643	70019
CN 634	7315		CN 644	43773
CN 635	19056		CN 645	79454
CN 636	19979		CN 646	229580
CN 637	2049		CN 647	43157
CN 638	3404		CN 648	29928
CN 639	12447		CN 649	84437
CN 640	4817		CN 650	88956
CN 641	16752		CN 651	74790
GMT+/-SEM	6391+/-1484			70378+/-12194

Example 4

Parenteral adjuvant activity of LT-K63 with HIV p24 gag in mice

a. Mice were immunized three times by subcutaneous injection 1 week apart with 10 μg LT-K63 (produced as described in Example 1) and 10 μg HIV p24

gag or 10 μg HIV p24 gag alone. HIV p24 gag-specific CTL activity is depicted in Table 4. CTL activity was measured in a standard chromium release assay and is presented as % specific lysis. In particular, SVBalb (H-2d) and MC57 (H-2b) target cells were incubated with 51Cr and luM p7g peptide for 1 hour. Effector cells (E) were cultured with target cells (T) at various E:T ratios for 4 hours. The average cpm from duplicate wells was used to calculate percent specific 51Cr release. Allogeneic Mc57 target cells and syngeneic SvBalb target cells had low background killing. Syngeneic SvBalb cells pulsed with HIV p24 gag epitope peptide p7g had 69% specific lysis at a 50:1 E:T ratio for HIV p24 gag with LT-K63. In contrast, HIV p24 gag alone induced only 29% killing under the same conditions. The group receiving LT-K63 had a higher CTL response in contrast to the gag alone group. This illustrates the adjuvant activity of LT-K63 for CTL induction with HIV p24 gag.

Table 4. CTL Responses of Mice Immunized with HIV gag and LT-K63K

		%specific lysis		
	Effector: Target	Mc57/p7g	SvB/p7g	SvB/-
LT-K63 + HIV p24 gag	50:1	2	69	7
	10	3	31	7
	2	4	14	4
HIV p24 gag	50:1	5	29	4
	10	3	12	2
	2	2	5	3

b. Mice were immunized twice by subcutaneous injection one month apart with 10 μg LT-K63 and 10 μg HIV

p24 gag. Sera were collected two weeks after the second immunization. The anti-HIV p24 gag titers are shown as geometric mean titer plus or minus standard error in Table 5. This experiment illustrates the ability of LT-K63 in combination with HIV p24 gag to produce an anti-HIV p24 gag response in mice.

Table 5. Antibody responses of mice immunized with p24 gag

Animal #	Day 0	Day 42
CP386	13	220406
CP387	15	153674
CP388	9	235706
CP389	20	350167
GMT+/-SEM		229900+/-38800

Example 5

Transcutaneous Adjuvant Activity of LT-K63 and LT-R72

For the following experiments, the LT wild-type (LTwt), LT-K63 and LT-R72 mutants were obtained as described (Giuliani et al., "Mucosal Adjuvanticity of LTR72, a Novel Mutant of *Escherichia coli* Heat-Labile Enterotoxin with Partial Knock-Out of ADP Ribosyltransferase Activity," *J. Exp. Med.* 187:in press). The antigen used, CRM_{197}, is a well-characterized non-toxic diphtheria toxin mutant. See, e.g., Bixler et al. (1989) *Adv. Exp. Med. Biol.* 251:175, Constantino et al. (1992) *Vaccine*; International Publication No. WO 96/14086.

For transcutaneous immunization, on day 0, groups of 5 female BALB/c mice were anesthetized with an intraperitoneal injection of 100 μl/10 g of weight of a solution of Ketavet™ 50 (20% v/v), Rompun™ (3% v/v), and

Combelen™ (3% v/v) in sterile saline. Mice were then shaved on the back (about 2 cm^2), and 100 μl of phosphate-buffered saline (PBS) containing 100 μg of CRM197 and 50 μg of LTwt or LT mutants were gently applied on the shaved skin. Mice were kept under anesthesia for about 1 hour, then washed with lukewarm tap water, and dried. The same procedure was repeated on day 21. Third and fourth immunizations were performed on day 51 and day 66, respectively. On the same dates, control groups of 5 mice received CRM197 (10 μg) and LTwt (1 μg) intranasally (20 μl volume).

Serum samples were taken at days -1, 20, 35, 65, and 80. Antibodies to LT and CRM were determined by standard ELISA procedures.

No anti-CRM antibody response was detectable. As shown in Table 6 and Figure 2, transcutaneous immunization induced a very strong anti-LT antibody response after one immunization (see Table 6), which was boosted after the second immunization (see Table 6 and Figure 2). Thus, transcutaneous immunization (i.e., application of soluble antigens plus mucosal adjuvants on the skin) induced the production of specific antibodies, showing that the immune system responded to the LT proteins. This result evidences that these proteins may be useful as transcutaneous adjuvants.

Table 6. Serum anti-LT antibody titers in BALB/c mice immunized transcutaneously

	mouse n.	day -1 (preimmune)	day 20 (post-1)	day 35 (post-2)	log titer day 35	mean	SD
Group 1 tc LT w.t.	1	*0	60856	81127	4.91		
	2	0	31319	833666	5.92		
	3	0	109229	256225	5.41	5.34	0.37
	4	0	129280	182907	5.26		
	5	0	35628	156077	5.19		
Group 2 tc CRM	6	0	0	0			
	7	0	0	0			
	8	0	0	0			
	9	0	0	0			
	10	0	0	0			
Group 3 tc CRM + LT w.t.	11	0	9593	99577	5.00		
	12	0	4606	73229	4.86		
	13	0	3455	60058	4.78	4.98	0.31
	14	0	5137	56589	4.75		
	15	0	20997	327216	5.51		
Group 4 tc CRM + LTK63	16	0	7691	16501	4.22		
	17	0	6307	37822	4.58		
	18	0	404	2770	3.44	3.90	0.49
	19	0	572	5382	3.73		
	20	0	843	3278	3.52		

Table 6. Serum anti-LT antibody titers in BALB/c mice immunized transcutaneously							
	mouse n.	day -1 (preimmune)	day 20 (post-1)	day 35 (post-2)	log titer day 35	mean	SD
Group 5 tc CRM + LTR72	21	0	2401	25676	4.41	4.67	0.30
	22	0	6868	45181	4.65		
	23	0	6868	33891	4.53		
	24	0	8049	38174	4.58		
	25	0	19452	186017	5.19		
Group 6 i.n. CRM + LT w.t.	26	0	169516	1195152	6.08	6.17	0.37
	27	0	104288	489685	5.69		
	28	0	210832	4000000	6.60		
	29	0	187184	989957	6.00		
	30	0	289546	3105000	6.49		

*0=negative (titer<50)

Groups 1 to 5 were immunized transcutaneously (tc), group 6 intranasally. For tc immunizations (days 0 and 21), groups of BALB/c mice were shaved on the back (about 2 cm^2) and kept under anesthesia for 1 hour. During this time, 100 microliters of PBS containing antigen CRM197 (100 micrograms) and LT or LT mutants (50 micrograms) were **applied on the shaved skin**. Mice were then extensively washed with lukewarm water to avoid possible oral intake of residual antigens. Serum samples were taken at the dates indicated, and tested by ELISA for quantitation of specific antibodies.

Thus, parenteral adjuvants comprising detoxified mutants of a bacterial ADP-ribosylating toxin are disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.